

# Essential role of Gata transcription factors in sympathetic neuron development

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## Summary

Sympathetic neurons are specified during their development from neural crest precursors by a network of crossregulatory transcription factors, which includes *Mash1*, *Phox2b*, *Hand2* and *Phox2a*. Here, we have studied the function of *Gata2* and *Gata3* zinc-finger transcription factors in autonomic neuron development. In the chick, *Gata2* but not *Gata3* is expressed in developing sympathetic precursor cells. *Gata2* expression starts after *Mash1*, *Phox2b*, *Hand2* and *Phox2a* expression, but before the onset of the noradrenergic marker genes *Th* and *Dbh*, and is maintained throughout development. *Gata2* expression is affected in the chick embryo by *Bmp* gain- and loss-of-function experiments, and by overexpression of *Phox2b*, *Phox2a*, *Hand2* and *Mash1*. Together with the lack of *Gata2/3* expression in *Phox2b* knockout mice, these results characterize *Gata2* as member of the *Bmp*-induced cluster

of transcription factors. Loss-of-function experiments resulted in a strong reduction in the size of the sympathetic chain and in decreased *Th* expression. Ectopic expression of *Gata2* in chick neural crest precursors elicited the generation of neurons with a non-autonomic, *Th*-negative phenotype. This implies a function for *Gata* factors in autonomic neuron differentiation, which, however, depends on co-regulators present in the sympathetic lineage. The present data establish *Gata2* and *Gata3* in the chick and mouse, respectively, as essential members of the transcription factor network controlling sympathetic neuron development.

Key words: Autonomic, Ciliary, Cholinergic, Noradrenergic, *Hand2* (dhand), *Bmp*, *Phox2b*, *Gata2*, *Gata3*, Chick, Mouse

## Introduction

Neural stem cells in the developing and adult nervous system have the potential to give rise to the different types of glial cells and to a large variety of distinct neuronal subtypes. Considerable progress has been made in the last few years in the identification of the molecular signals and mechanisms that control neuronal and glial specification and differentiation. In particular, the generation of autonomic neurons from neural crest stem cells was shown to be induced by an extrinsic signal (*Bmps*), which elicits the expression of a network of transcription factors that, in turn, control autonomic neuron differentiation (Goridis and Rohrer, 2002). This network includes *Mash1* (*Ascl1* – Mouse Genome Informatics), the mammalian homologue of the *Drosophila* achaete scute gene complex, and the paired homeodomain transcription factors *Phox2b*, possibly acting in concert with their coexpressed paralogue *Phox2a*. *Phox2* proteins bind to the promoter of the subtype-specific noradrenergic marker genes tyrosine hydroxylase (*Th*) and dopamine- $\beta$ -hydroxylase (*Dbh*) and activate their transcription.

Although *Mash1* and *Phox2b* were shown to be essential and

sufficient to elicit noradrenergic neuron development from neural crest precursor cells, they also function together in the generation of cholinergic autonomic neurons and several other neuron subtypes. Thus, to generate noradrenergic neurons, *Mash1* and *Phox2* genes must be enforced by the action of additional autonomic and noradrenergic regulators expressed in this lineage. The basic helix-loop-helix (bHLH) transcription factor *Hand2* (previously known as dHand) has recently been identified as a noradrenergic co-determinant, due to its ability to elicit noradrenergic differentiation in neural crest (Howard et al., 2000) and parasympathetic precursors (Müller and Rohrer, 2002), and due to its expression in noradrenergic sympathetic but not in parasympathetic ciliary neurons (Müller and Rohrer, 2002). The effects on the expression of the noradrenergic marker gene dopamine- $\beta$ -hydroxylase (*Dbh*) can be explained by a direct interaction with *Phox2a* to stimulate transcription from the *Dbh* promoter (Xu et al., 2003; Rychlik et al., 2003). Finally, members of the *Gata* family of transcription factors have been implicated in the control of noradrenergic differentiation (Groves et al., 1995; Lim et al., 2000).

The *Gata* transcription factors are key regulators of

hematopoiesis (Pevny et al., 1991; Tsai et al., 1989; Maeno et al., 1996; Murphy and Reiner, 2002), cardiovascular and urogenital development (Zhou et al., 1998; Molkentin et al., 1997) and nervous system development (Pandolfi et al., 1995; Nardelli et al., 1999; Pata et al., 1999; Dasen et al., 1999; van Doorninck et al., 1999; Craven et al., 2004; Karis et al., 2001). In hematopoiesis and developing heart and liver, Gata transcription factors mediate the effects of Bmps (Maeno et al., 1996; Schultheiss et al., 1997; Rossi et al., 2001). Recent evidence suggests that Gata factors maintain Bmp expression during cardiac precursor maturation (Peterkin et al., 2003; Klinedinst and Bodmer, 2003). Gata factors are characterized by two zinc finger domains that mediate binding to a DNA motif centred around the nucleotide sequence GATA (Yamamoto et al., 1990; Ko and Engel, 1993; Whyatt et al., 1993). The Gata family is composed of six vertebrate family members that are expressed in distinct spatiotemporal patterns. However, of all family members, only Gata2 and Gata3 are present in the nervous system, where their expression overlaps extensively (Kornhauser et al., 1994; Nardelli et al., 1999; Pata et al., 1999). The analysis of mice deficient for *Gata2* or *Gata3* revealed that Gata2 controls Gata3 expression in many, but not all expression domains (Nardelli et al., 1999; Pata et al., 1999). In the central nervous system, Gata2 is essential and sufficient for spinal cord interneuron generation (Zhou et al., 2000; Karunaratne et al., 2002), for the induction of ventral gonadotrope and thyrotrope fates in the pituitary (Dasen et al., 1999) and for the generation of serotonergic neurons in rostral hindbrain (Craven et al., 2004). *Gata3* was shown to be involved in the development of serotonergic neurons in the caudal raphe nuclei (van Doorninck et al., 1999; Pattyn et al., 2004), in ear formation (Karis et al., 2001; Lawoko-Kerali et al., 2002), and in the expression of the noradrenergic marker genes *Th* and *Dbh* in the peripheral nervous system (Lim et al., 2000).

Since in the sympathetic ganglia of *Gata3*-deficient mice abrogation of *Th* and *Dbh* expression, but normal generic neuronal differentiation, has been reported (Lim et al., 2000), Gata3 was considered to selectively control neuron subtype differentiation and to represent a noradrenergic co-determinant for *Phox2a/b* and *Mash1*. Although the *Gata3* knockouts demonstrated the importance of this factor, its position in the transcriptional network specifying sympathetic neurons was not clear. Here, we have analysed the action of Gata transcription factors in autonomic neuron development in the chick and re-investigated the sympathetic neuron phenotype in *Gata3*-deficient mouse embryos. We demonstrate that *Gata2* but not *Gata3* is expressed in the avian autonomic nervous system. *Gata2* expression in the chick starts after the expression of *Cash1*, *Phox2b*, *Phox2a* and *Hand2* and is induced by overexpression of these transcription factors. Bmp-dependent expression characterizes *Gata2* as an additional member of the transcriptional network acting in the sympathetic lineage downstream of Bmps. The elimination of *Gata3* in the mouse and the knockdown of *Gata2* in the chick result in a strong decrease in both sympathetic ganglion size and *Th* expression. These results, together with the effect of *Gata2* overexpression demonstrate a function for *Gata2/3* in the type-specific, as well as generic, differentiation of noradrenergic neurons, acting in the context of other autonomic co-determinants.

## Materials and methods

### Expression pattern of *Gata* transcription factors

Chick embryos were staged according to Hamburger and Hamilton (1951). Embryos between embryonic day (E) 3 (stage 18) and E20 (stage 45) were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight. The fixative was replaced by 15% sucrose in 0.1 M sodium phosphate buffer overnight. Cryosections of 12  $\mu$ m were analysed for expression of *Phox2b*, *Hand2*, *Phox2a*, *Gata2*, *Gata3*, *Th* and *Dbh* by in-situ hybridisation. At least three embryos were analysed for each stage.

### Implantation of agarose beads loaded with noggin or BSA in chick embryos

The implantation technique used is described in detail by Schneider et al. (Schneider et al., 1999). Agarose beads (Affi-Gel blue beads; Biorad, Hercules, CA) were incubated for at least 1 hour in a small volume of loading buffer containing either 1 mg/ml noggin or bovine serum albumin (BSA). Two beads were implanted into the trunk region of 2-day-old chick embryos, placed at the last somite and 2-3 somites more rostral. The eggs were further incubated until stage 19, fixed, embedded and sectioned. Cryosections of 12  $\mu$ m were collected, including from the implantation area, and analysed for expression of *Sox10* and *Gata2* by in-situ hybridisation. The area of *Sox10* and *Gata2* expression was quantified morphometrically and the areas were expressed in  $\mu$ m<sup>2</sup>/section. The results are given as the mean area per section  $\pm$  s.e.m. of at least six embryos analysed.

### Expression of transgenes in vivo using retroviral replication-competent avian sarcoma (RCAS) vectors

Fertilized virus-free chicken eggs were obtained from Charles River (Sulzfeld, Germany) and incubated for 2 days. Cell aggregates of DF1 fibroblasts infected with RCASBP(B)-*Hand2* (Howard et al., 2000), RCASBP(B)-*Phox2b* (Stanke et al., 1999), RCASBP(B)-CNS-*Gata2*, RCASBP(B)-CNS-dn*Gata2*, RCASBP(B)-CNS-*engrailed* and RCASBP(B)-CNS-VP16-*Gata2* were implanted on the right site of the embryos at brachial levels between the neural tube and the last somite formed (Reissman et al., 1996). The eggs were further incubated until E8. Embryos were killed by decapitation. The trunk and cervical region of the embryos were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight, kept in 15% sucrose in 0.1 M sodium phosphate buffer overnight, embedded in Tissue Tek (Sakura Finetek Europ BV, Zoeterwoude, The Netherlands) and sectioned. Cryosections of 12  $\mu$ m were collected and analysed for expression of reverse transcriptase (*RT*), *Th*, *Scg10* and *Gata2* (RCASBP(B)-*Hand2* and RCASBP(B)-*Phox2b* infections); *RT*, *Th*, *Scg10*, *NF160*, *Phox2b* and *Cash1* (RCASBP(B)-CNS-*Gata2* infections) and *RT*, *Th*, *Dbh*, *Scg10*, *NF160* and *Phox2b* (RCASBP(B)-CNS-dn*Gata2*-, RCAS-BP(B)-CNS-*engrailed* and RCASBP(B)-CNS-VP16-*Gata2* infections) by in-situ hybridisation. For the quantitative analysis, between 5 and 13 embryos were analysed for each of the genes investigated.

### Mouse breeding, genotyping and rescue

The generation and genotyping of *Phox2b* mutant mice and *Gata3* mutant mice have been reported (Pattyn et al., 1999; Pandolfi et al., 1995; Lim et al., 2000). Homozygous *Gata3* mutants, which normally die at mid-gestation, were rescued beyond E10 with noradrenergic agonists as described for *Phox2b* mutants (Pattyn et al., 2000).

### In-situ hybridisation on sections

Non-radioactive in-situ hybridisation on cryosections and preparation of digoxigenin- or fluoresceine-labelled probes for chick *RT*, *Th*, *Scg10*, *NF160*, *Gata2*, *Gata3*, *Phox2b*, *Phox2a*, *Hand2*, *Cash1* and *Sox10* were carried out as described previously (Ernsberger et al., 1997; Stanke et al., 1999) (*Gata2* and *Gata3* plasmids were generously provided by M. Zenke). For double in-situ hybridisations

Fast Red (Roche Diagnostics, Mannheim, Germany) was used for staining the first probe. Sections were photographed and the antibody was stripped off by washing twice for 10 minutes with 1 ml 0.1 M glycine pH 1.8. After equilibration in MABT for 1 hour, the second colour reaction with Nitroblue Tetrazolium/S-bromo- $\Delta$ -chloro-3-indolyl phosphate (NBT/BCIP) was carried out.

In-situ hybridisation using mouse *Dbh*, *Gata2* (gift of J. Nardelli), *Gata3* (gift of D. Engel), *Hand2* (gift of Y.-S. Dai), *Mash1* (gift of F. Guillemot), *Ret*, *Sox10* (gift of K. Kulbrodt) and *Th* antisense riboprobes, immunohistochemistry using Phox2a, Phox2b,  $\beta$ -galactosidase (Cappel) antisera or Tuj1 monoclonal antibody (Covance), and combined in-situ hybridisation with immunohistochemistry were performed as previously described (Tiveron et al., 1996). Double-immunofluorescence experiments using Phox2a and Tuj1 antibodies were analysed on a Leica microscope. Pictures were superimposed in Photoshop.

### Morphometric analysis

#### Chick

The area of *Th*, *Dbh*, *Phox2b*, *NF160* and *Scg10* expression was quantified morphometrically using the Metamorph Imaging System (version 4.6, Universal Imaging Corporation) on all sections infected by the virus, as indicated by expression of RT mRNA. Areas were expressed in  $\mu\text{m}^2/\text{section}$ . The results are given as the mean area per section  $\pm$  s.e.m. of at least five embryos analysed. Student's *t*-test was used for statistical analysis.

#### Mouse stellate ganglion

The surface of the stellate ganglion – stained with Phox2b antibody – has been calculated on saggital sections of E13.5 embryos using the Leica Qfluoro Program. Measurements of control ganglia were considered as 100%. For each genotype, four sections were counted on four chains corresponding to two embryos.

#### Mouse thoracic chain

The number of Phox2b-positive cells were counted on saggital sections at E13.5 at the thoracic level on a segment spanning three vertebrae, at the same level in the control and the mutants. For each genotype, four sections were counted on four chains corresponding to two embryos.

### TUNEL analysis

TUNEL-positive cells were detected using the apoptag detection kit (Appligene) following the manufacturer's instructions. The rostralmost part of the sympathetic chain (anterior to the fusion of the dorsal aorta) was delimited on transverse sections at E11.5 using a *Sox10* in-situ hybridisation signal on adjacent sections, and cells were counted within that area. For each genotype, six sections were analysed on four chains corresponding to two embryos.

### Construction of plasmid transgenes

#### RCAS-BP(B)-CNS-Gata2

PCR technology was used to insert a Kozak sequence linked to a *ClaI* site and a *NotI* site flanking the coding sequence of ggGata2 (Yamamoto et al., 1990). Primer: sense: 5'-AGT **ATC GAT GAC CAC C AT G** GA GGT GGC CAC GGA TCA GC-3'; antisense: 5'-GAT CGA **GCG GCC GC T TA** T CCC ATG GCT GTA ACC AT-3'. (sense primer: bold, *ClaI* site; underlined, Kozak sequence; bold+underlined, Start) (antisense primer: bold, *NotI* site; bold+underlined, Stop)

The PCR product was then cloned directly into the pCRII-TOPO vector (Invitrogen). After restriction analysing and sequencing, the insert was cloned into the *ClaI* and *NotI* sites of the avian retroviral vector RCAS-BP(B)-CNS. The RCAS-BP(B)-CNS is a modification of the RCAS-BP(B) vector (Hughes and Kosik, 1984), inserting a unique *NotI* and *SpeI* site directly behind *ClaI*<sup>(7029)</sup>.

#### RCAS-BP(B)-CNS-dnGata2

PCR technology was used to insert a *BamHI* site and an *XbaI* site flanking the two zinc finger domains of ggGata2 (ggGata2 S<sup>276</sup>-I<sup>379</sup>, equivalent to hsGata3 S<sup>258</sup>-I<sup>361</sup>) (Yamamoto et al., 1990; Yang et al., 1994). Primer: sense: 5'-CTG **GGA TCC TCA GAA GGC AGA GAG TGT GTG AA**-3'; antisense: 5'-TTC **TCT AGA TTC GTT TTT CAT GGT CAG AGG CC**-3'. (sense primer: bold: *BamHI* site; antisense primer: bold: *XbaI* site)

The *BamHI-XbaI* digested PCR fragment was ligated into the pIEP vector (pIEP vector was generously provided by C. Goriadis) after eliminating the original *BamHI-XbaI* fragment (Phox2a DNA binding site). The original pIEP vector contains the engrailed effector domain from *Drosophila melanogaster* (AA<sup>1</sup>-AA<sup>298</sup>) (Han and Manley, 1993) upstream from the Phox2a homeodomain, that is flanked by a *BamHI* site and an *XbaI* site. The pIEP vector also contains two myc-tags downstream from the *XbaI* site. PCR technology was then used to insert a Kozak sequence linked to a *ClaI* site and a *SpeI* site flanking the dnGATA2 coding region. The PCR product was cloned into the *ClaI* and *NotI* site of the RCAS-BP(B)-CNS vector. Primer: sense: 5'-ACA **ATC GAT GCC GCC A AT G** GC CCT GGA GGA TCG CTG CA-3'; antisense: 5'-TCT **ACT AGT TCA** CAG GTC CTC CTC GCT GAT CAG-3'. (sense primer: bold, *ClaI* site; underlined, Kozak sequence; bold+underlined, start) (antisense primer: bold, *SpeI* site; bold+underlined, stop)

#### RCAS-BP(B)-CNS-VP16-Gata2

The engrailed domain of RCAS-BP(B)-CNS-dnGata2 was substituted by the VP16 domain (Ala<sup>481</sup>-Gly<sup>541</sup> + spacer of 6 AA) (VP16 vector was generously provided by C. Goriadis).

#### RCAS-BP(B)-CNS-engrailed

The zinc finger domain of RCAS-BP(B)-CNS-dnGata2 was substituted by the spacer Ala-Gly-Gly.

### Semiquantitative RT-PCR analysis

Total RNA from chick sympathetic ganglia was isolated by using an RNeasy kit (Qiagen). Relative levels of *Gata2* and *Gata3* expression were determined by RT-PCR. Primer pairs were designed for amplification of specific cDNA fragments.

*Gata2* primers: sense: 5'-CAA CTA CAT GGA ACC AGC GC-3'; antisense: 5'-AGG CTG CTG CTG TAG TCA TG-3'; *Gata3* primers: sense: 5'-CTC CGT ATT ACG GCA ACT CC-3'; antisense: 5'-GCT GCA GAC AGC CTT CTC TT-3'.

cDNA from total RNA was synthesised with oligo(dT) primers and Moloney murine leukaemia virus reverse transcriptase (Superscript II; Life Technologies) at 45°C for 1 hour. cDNA derived from 20-30 ng RNA was used as template for PCR amplification in a 50  $\mu\text{l}$  reaction volume containing 1 $\times$  PCR buffer, 0.2 mM dNTPs and 0.1  $\mu\text{M}$  each primer. Hot start was performed by adding 1.5 units of AmpliTaq. The temperature profile consisted of 25-36 cycles (95°C for 15 seconds, 65°C for 30 seconds and 72°C for 30 seconds) and a final 5 minutes extension at 72°C. To achieve accurate quantification, 10  $\mu\text{l}$  aliquots were collected during the PCR run at various cycle numbers. PCR products were separated by electrophoresis on 1% agarose gel and stained with Ethidiumbromide. Their fluorescence intensities were measured by using the Gel Doc 2000 (Bio Rad Laboratories). In all experiments, amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA fragments was run in parallel to normalize different cDNA samples (Friedel et al., 1997).

## Results

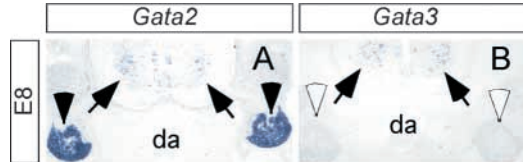
### Expression of *Gata2* and *Gata3* in the chick and mouse sympathetic lineage

In the vertebrate peripheral nervous system, *Gata3* expression has been analysed only in the mouse embryo (George et al.,

1994), whereas data on *Gata2* expression are available only for autonomic neurons in the trunk of chick embryos (Groves et al., 1995). The present in-situ hybridisation analysis of *Gata2* and *Gata3* expression in the chick embryo demonstrated that in sympathetic ganglia analysed between E3 (stage 18) and E20 (stage 45), *Gata2* but not *Gata3* was detectable (Fig. 1). This result was confirmed by semiquantitative RT-PCR, which showed about 1000-fold lower levels of *Gata3* mRNA compared with *Gata2* mRNA in E7 sympathetic ganglia (the mean of two experiments). By contrast, *Gata2* and *Gata3* are coexpressed in the spinal cord (Fig. 1), as described previously (Kornhauser et al., 1994; Nardelli et al., 1999; Pata et al., 1999). This finding suggests that, in the chick, *Gata2* rather than *Gata3* may be involved in noradrenergic differentiation. By contrast, in the mouse embryo, *Gata2* and *Gata3* are expressed at equivalent levels in sympathetic ganglia (Fig. 5).

### ***Gata2* is expressed after the onset of *Phox2a*, *Phox2b* and *Hand2* expression**

We analysed the developmental expression of Gata factors in the sympathetic lineage of the chick embryo in relation to other developmental marker genes as a first indication of their epistatic relationship. Chick embryos (stage 18-19) were sectioned in the brachial region and analysed by in-situ hybridisation on consecutive sections. Embryos were grouped according to the number of somites. In 32-somite embryos, *Gata2* was not yet detected, whereas *Phox2b* was strongly expressed and *Hand2* expression just started (Fig. 2). At this



**Fig. 1.** Expression of *Gata2* and *Gata3* in chick sympathetic ganglia. Frozen sections at the brachial level of E6 chick embryos were analysed for the expression of *Gata2* and *Gata3* by in-situ hybridisation. Sympathetic ganglia display a strong hybridisation signal for *Gata2* (black arrowheads), whereas *Gata3* expression was not detected (white arrowheads). In the spinal cord both *Gata2* and *Gata3* are expressed (black arrows). da, dorsal aorta.

axial level, *Gata2* expression in primary sympathetic ganglia was first detected in 33/34-somite embryos, i.e. after *Phox2b*, *Hand2* and *Phox2a*, but before the onset of *Th* expression, which began in 35-somite embryos (Fig. 2). In addition to sympathetic ganglion primordia, *Gata2* is also expressed in the ventral part of the dorsal aorta. The results, summarized in Table 1, show that *Gata2* is expressed before the noradrenergic marker genes, which is a prerequisite for the proposed function in noradrenergic differentiation.

### ***Gata2* expression is dependent on Bmp signalling**

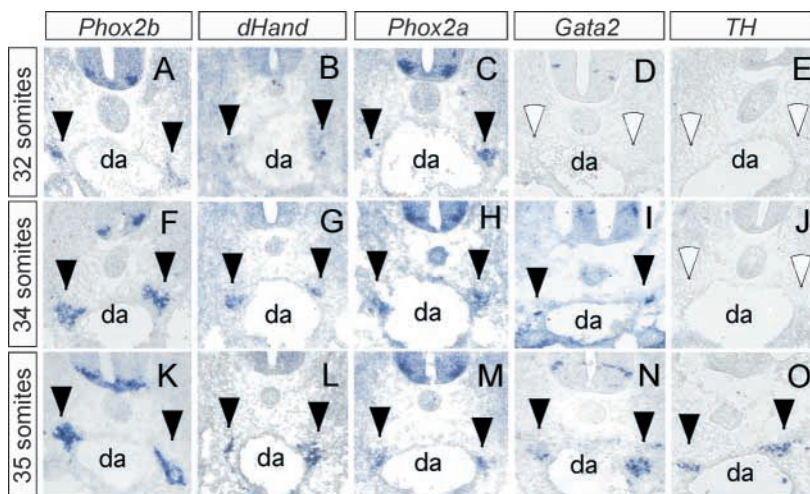
Previous studies demonstrated that the expression of *Mash1*, *Phox2a*, *Phox2b* (Schneider et al., 1999) and *Hand2* (Howard et al., 2000) is dependent on Bmps that are expressed in the dorsal aorta, close to the developing sympathetic ganglia. To investigate whether *Gata2* expression would be controlled also by Bmps or other, unknown signals, the Bmp antagonist noggin was applied in vivo in the area where the sympathetic ganglia form. Using *Sox10* as a general marker for neural crest and early neural crest derivatives, only a small reduction in the number of sympathetic precursors, located dorsolaterally from the dorsal aorta, was apparent (Fig. 3). By contrast, *Gata2* expression was strongly reduced or absent. The effect of the noggin treatment was restricted to the primordia of the sympathetic ganglia and did not affect *Gata2* expression in the spinal cord or ventral aorta. Quantification by the

**Table 1.** Expression of *Phox2b*, *Hand2*, *Phox2a*, *Gata2*, *Th* and *Dbh* gene transcripts in the chick embryo

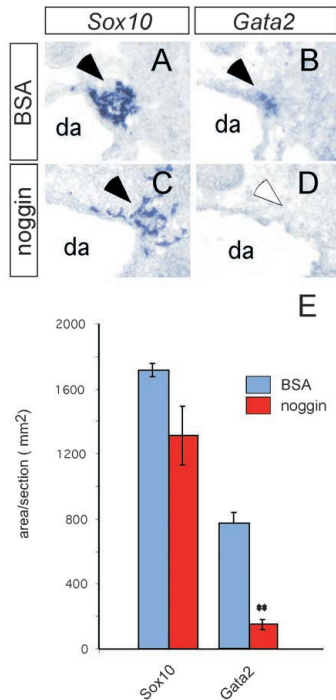
Hamburger/Hamilton stage 18/19						
Somites	29-30	31-32	33-34	35-36	36-37	
Probe						
<i>Phox2b</i>	+, +, +	+, +, +, +	+, +, +, +, +	+, +, +	+, +, +	
<i>Hand2</i>	-, -, -	-, +, +, +	+, +, +, +, +	+, +, +	+, +, +	
<i>Phox2a</i>	-, -, -	-, -, -, +	+, +, +, +, +	+, +, +	+, +, +	
<i>Gata2</i>	-, -, -	-, -, -, -	-, +, +, +, +	+, +, +	+, +, +	
<i>Th</i>	-, -, -	-, -, -, -	-, -, -, -, -	+, +, +	+, +, +	
<i>Dbh</i>	-, -, -	-, -, -, -	-, -, -, -, -	-, +, +	+, +, +	

All sections examined correspond to the wing bud region. Data from one embryo are listed in one row.

+, sections with positive cells in primary sympathetic ganglia; -, sections devoid of positive cells in primary sympathetic ganglia.



**Fig. 2.** The onset of *Gata2* expression in sympathetic ganglia in relation to the expression of *Phox2b*, *Hand2*, *Phox2a* and *Th*. Frozen sections from the brachial region of stage 18/19 chick embryos, staged according to the number of somites, were analysed for the expression of *Phox2b*, *Hand2*, *Phox2a*, *Gata2* and *Th*. *Gata2* expression was first observed in 34-somite embryos, after the onset of expression of *Phox2b*, *Hand2* and *Phox2a*, but before *Th*, which was first observed in 35-somite embryos. Black and white arrowheads indicate the presence or absence, respectively, of gene expression in primary sympathetic ganglia. *Gata2* expression was additionally detected in the ventral part of the dorsal aorta (da) (I) and in the spinal cord (D,I,N). The results are summarised in Table 1.

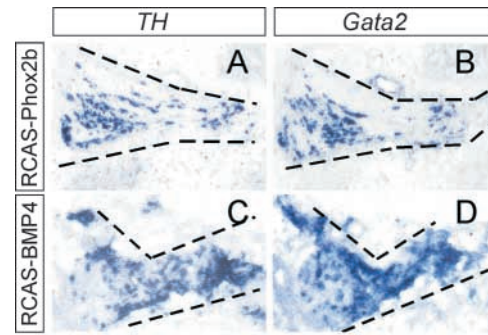


**Fig. 3.** *Gata2* expression in sympathetic ganglion primordia is blocked by the *Hand2* inhibitor *noggin*. Chick embryos were treated with BSA (A,B) or *noggin* (C,D) at stage 14 and analysed at stage 19 for the expression of *Sox10* (A,C) and *Gata2* (B,D) in sympathetic ganglion primordia close to the dorsal aorta (da). Arrowheads point to primary sympathetic ganglia. (E) *Sox10* and *Gata2* expression is quantified by determining the area of *Sox10*- and *Gata2*-expressing cells. The mean $\pm$ s.e.m. of at least three embryos is shown. \*\* significantly different from BSA controls ( $P < 0.01$ ) (Student's *t*-test).

morphometric analysis of *Sox10*- and *Gata2*-positive cells revealed a strong, 80% reduction in *Gata2* expression and a non-significant reduction (24%) in *Sox10* expression. Since the number of *Sox10*-positive cells is reduced only to a small extent, the effect on *Gata2* must reflect the action of *Bmps* on *Gata2* expression rather than on the survival of *Gata2*-positive cells. In agreement with these loss-of-function experiments, strong *Gata2* expression was also detected in the *Bmp*-induced ectopic neurons in peripheral nerve (Fig. 4C,D).

### Epistatic relationship between *Gata2*, *Phox2b* and *Hand2*

As *Gata2* is expressed after the *Bmp*-induced factors *Phox2b*, *Hand2* and *Phox2a*, it seemed very likely that the effect of *Bmps* is indirect, mediated by these transcription factors. To address the epistatic relationship between *Gata2* and *Phox2b* and other *Bmp* downstream transcriptional regulators, both gain- and loss-of-function approaches were followed. Previous studies demonstrated that *Phox2b* and *Hand2* expression in the brachial nerve results in the generation of ectopic *Th*<sup>+</sup> and *Dbh*<sup>+</sup> noradrenergic neurons (Stanke et al., 1999; Howard et al., 2000). Using this experimental paradigm, we investigated whether *Gata2* would be expressed under these conditions. On consecutive sections of *Phox2b*-infected brachial nerve (Fig. 4)



**Fig. 4.** *Gata2* is induced in peripheral nerve precursors by ectopic expression of *Phox2b* and *BMP4*. Chick embryos were infected at E2 with RCAS-*Phox2b* and RCAS-*BMP4* and analysed at E8 for the presence of ectopic neurons in the brachial nerve. In response to *Phox2b* and *BMP4* neurons were generated that express noradrenergic properties: *Th* (A,C) and *Gata2* (B,D). These cells also coexpress neuronal characteristics (Stanke et al., 1999; Howard et al., 2000).

was observed, strongly suggesting that *Gata2* is induced by *Phox2b*. Similarly, *Gata2* and *Th* expression were induced in response to *Mash1* and *Hand2* (not shown), although the number of ectopically induced cells was lower compared with *Phox2b*. Since frequent reciprocal crossregulations in the sympathetic lineage make it difficult to rigorously establish epistatic relationships by overexpression experiments (Stanke et al., 1999, 2004; Howard et al., 2000), *Gata2/3* expression was investigated in *Phox2b* knockout mice. The absence of *Gata2* and *Gata3* expression in E10.5 sympathetic ganglion primordia (Fig. 5) confirmed our conclusion that *Gata2/3* acts downstream of *Phox2b* in the network of *Bmp*-induced transcription factors.

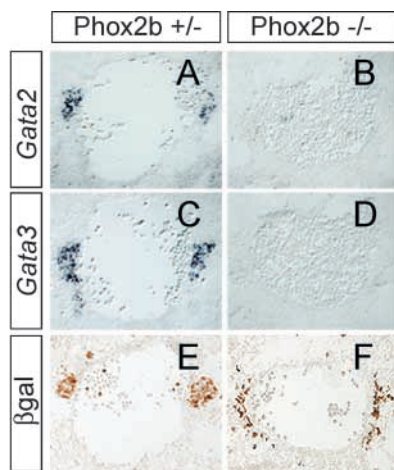
### Essential role of *Gata2* in chick sympathetic neuron generation

The strong effects of the *Gata3* knockout on noradrenergic gene expression in the mouse (Lim et al., 2000) raised the question whether elimination of *Gata2*, which in the chick sympathetic lineage appears to be functionally equivalent to mouse *Gata3*, would also impair the development of the noradrenergic phenotype. To interfere with the action of endogenous *Gata2*, a repressive form of *Gata2*, in which the engrailed repressor domain is fused to *Gata2* zinc finger domains, was expressed in sympathetic neuron precursor cells. Previous studies have demonstrated that the engrailed repressor does not function by titrating promoter binding sites but rather interferes with transcription initiation (Han and Manley, 1993; Jaynes and O'Farrell, 1991). Indeed, a similar engrailed-*Gata2* fusion protein has very recently been constructed and was shown to act as dominant-negative *Gata2* (dn*Gata2*) (Craven et al., 2004). Dn*Gata2* was expressed unilaterally using RCAS retroviral vectors, so that the sympathetic ganglia of the contralateral side could be used as internal control. As additional controls, ganglia were infected with RCAS vectors expressing only the engrailed repressor domain or a VP16-*Gata2* variant, where the transactivating N-terminal region of *Gata2* is replaced by the VP16 activation domain. The expression of dn*Gata2* in sympathetic neuron precursors resulted in a strong (50%) reduction of *Th* expression (Fig. 6),

and smaller effects on *Scg10* (*Stmn2* – Mouse Genome Informatics) (35%), *Dbh* (41%) and *Phox2b* (31%) (Fig. 6). The expression of neurofilament (NF 160) was also strongly reduced (not shown). All reductions were highly significant ( $P < 0.01$ ) with respect to the control side. *Th* expression was also significantly reduced compared with *Phox2b* ( $P < 0.05$ ;  $n = 11$ ) and *Scg10* ( $P < 0.05$ ;  $n = 11$ ), whereas *Dbh* was not significantly more reduced than *Phox2b* and *SCG10*. Control infections with the engrailed RCAS virus or RCAS-VP16-GATA2 affected neither *Th* nor *Scg10* expression (Fig. 6), excluding the possibility that dnGata2 would act in an unspecific manner by titrating transcription factors unrelated to endogenous Gata2. In conclusion, these results demonstrate a dual effect of Gata2 knockdown: a reduction in sympathetic ganglion size, reflected by a smaller area of *Phox2b*- and *SCG10*-expressing cells, and an additional effect on *Th* expression in the remaining cells. These effects suggest a more general role in sympathetic neuron development than reported for murine Gata3 (Lim et al., 2000).

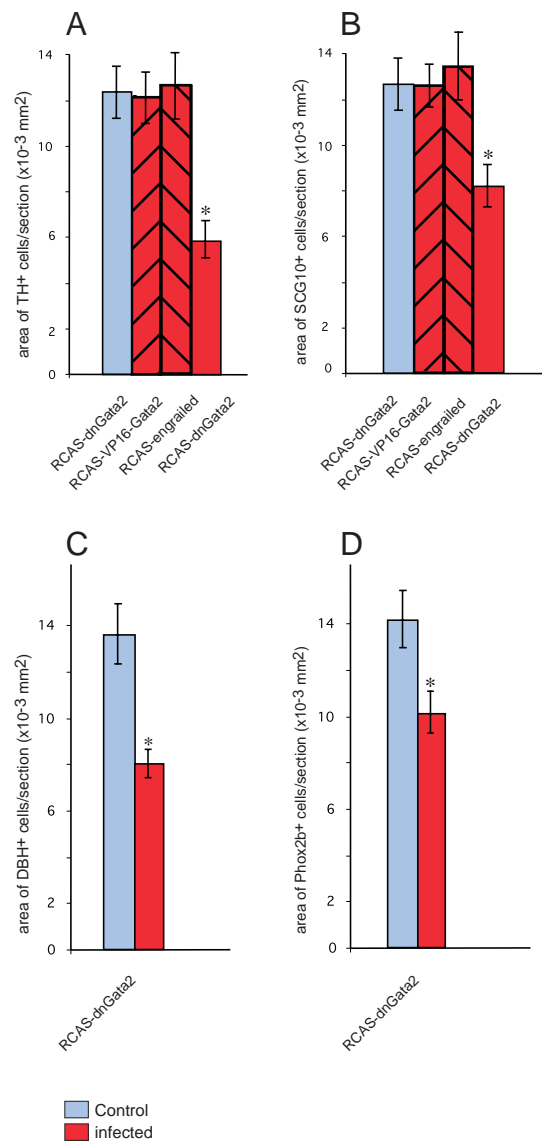
### Gata3 knockout mice display strong defects in sympathetic neuron development

To resolve the different results obtained by interfering with Gata2 and Gata3 in the chick and mouse, respectively, the development of the sympathetic lineage was re-investigated in *Gata3*<sup>-/-</sup> mice. In the *Gata3* knockout, sympathetic ganglion primordia of normal size are present at E10.5 (Fig. 7), as demonstrated by the expression of transcriptional control genes (*Mash1*, *Phox2b*, *Phox2a*, *Hand2*), noradrenergic markers (*Dbh*, *Th*), the GFL receptor signalling subunit (*Ret*) and generic neuronal markers (*Tuj1*). The only differences to control ganglia were a lack of *Gata2* expression and substantially lower *Th* expression levels, whereas *Dbh* expression was intact (Fig. 7). By contrast, E11.5 sympathetic ganglia were reduced in size in *Gata3*-deficient animals (Fig. 8). This can be explained by a strong increase in the number



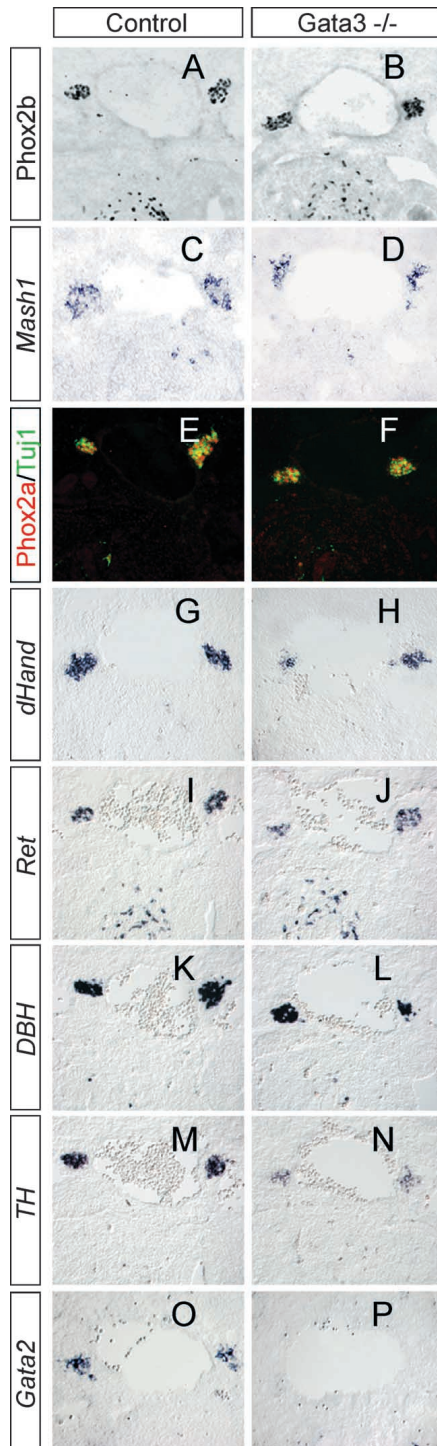
**Fig. 5.** Lack of *Gata2* and *Gata3* expression in *Phox2b*<sup>-/-</sup> mice. The expression of *Gata2* and *Gata3* was studied in E13.5 *Phox2b*<sup>LacZ/+</sup> and *Phox2b*<sup>LacZ/LacZ</sup> mouse embryos. In the *Phox2b* knockout *Gata2* (B) and *Gata3* (D) were not detectable, by contrast to the heterozygotes (A,C). Sympathetic ganglion precursors could be detected at the dorsal aorta in both *Phox2b*<sup>+/-</sup> (E) and *Phox2b*<sup>-/-</sup> (F) embryos by LacZ staining.

of apoptotic, TUNEL-positive cells observed at this stage (Fig. 8). At E13.5, the oldest stage analysed, only rudiments of the stellate ganglion and the superior cervical ganglion (SCG) (not

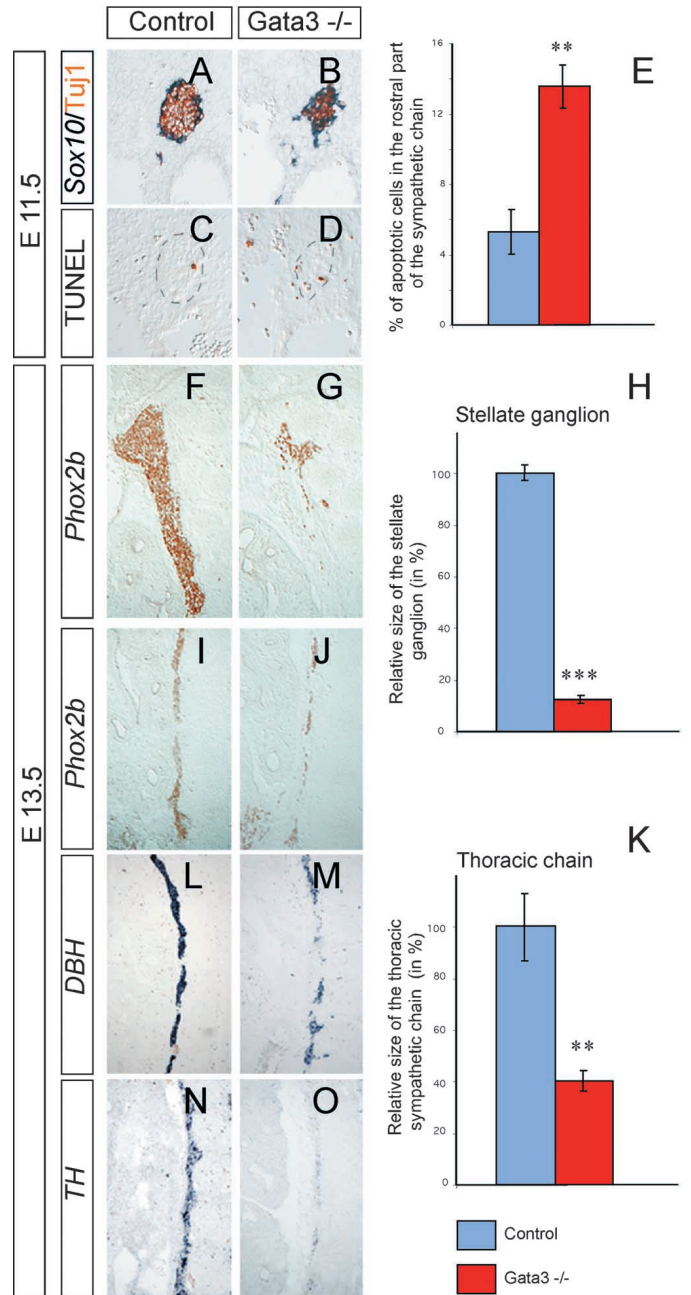


**Fig. 6.** The expression of a dominant-negative variant of Gata2 results in a reduction of *Th*, *Scg10*, *Dbh* and *Phox2b* expression. Chick embryos were infected unilaterally at E2 with either RCAS-dnGata2 or RCAS-engrailed and RCAS-VP16-Gata2 as controls and analysed at E8 for the expression of *Th* (A), *Scg10* (B), *Dbh* (C) and *Phox2b* (D) in infected sympathetic ganglia. The areas of *Th*-, *Scg10*-, *Dbh*- or *Phox2b*-expressing cells were quantified on alternate sections, both on the infected and the contralateral uninfected side. For the RCAS-engrailed and RCAS-VP16-Gata2 controls, only data of the infected ganglia are shown for simplicity; the data of the uninfected side are identical. The data represent the mean ± s.e.m. of at least nine embryos. (A) \* significantly different from engrailed-infected side, VP16-Gata2-infected side and dnGata2-uninfected side ( $P < 0.001$ ,  $P < 0.005$ ,  $P < 0.005$ , respectively). (B) \* significantly different from engrailed-infected side, VP16-Gata2-infected side and dnGata2-uninfected side ( $P < 0.005$ ,  $P < 0.01$ ,  $P < 0.01$ , respectively). (C) \* significantly different from dnGata2-uninfected side ( $P < 0.005$ ). (D) \* significantly different from dnGata2-uninfected side ( $P < 0.01$ ) (all Student's *t*-tests).

shown) were detectable in *Gata3*<sup>-/-</sup> embryos by analysing Phox2b expression (Fig. 8). At more caudal levels, the effect on ganglion size was less dramatic, but the thoracic chain was still reduced by 60% in size. In the remaining cells in the



**Fig. 7.** Gene-expression pattern in E10.5 sympathetic ganglia of *Gata3*<sup>-/-</sup> mice. The expression of Phox2b (A,B), Mash1 (C,D), Phox2a/Tuj1 (E,F), Hand2 (G,H), Ret (I,J) and Dbh (K,L) is not affected in *Gata3*-deficient mice. By contrast, the ganglia are devoid of *Gata2* expression (O,P) and display a significant reduction in Th expression levels (M,N).



**Fig. 8.** Reduction in ganglion size and Th expression and increased apoptosis in sympathetic ganglia of *Gata3*<sup>-/-</sup> mice.

(A,B) Immunostaining for  $\beta$ III-tubulin combined with in-situ hybridisation for *Sox10* reveal a reduced ganglion size in mutant (B) compared with control (A) embryos at E11.5. (C,D) Cell death analysis by TUNEL staining showing an increased number of apoptotic cells. (E) Quantification ( $P < 0.01$ ;  $n = 4$ ). (F-O) Reduction in sympathetic ganglion size and Th expression in E13.5 sympathetic ganglia of *Gata3*<sup>-/-</sup> mice. In *Gata3*<sup>-/-</sup> mice the sympathetic stellate ganglion (G) is strongly reduced in size compared with wild type (F), as revealed by immunostaining for Phox2b antibody. The reduction, quantified in (H) is by about 88% ( $P < 0.001$ ;  $n = 4$ ). Also at thoracic levels (I,J,L,M) a strong reduction in ganglion size is evident on Phox2b immunostains (I,J), quantified in (K) at around 60% ( $P < 0.01$ ;  $n = 4$ ). While *Dbh* expression was somewhat reduced (L,M), *Th* expression was almost undetectable in the mutants (N,O).

ganglion rudiments, *Th* expression was practically abolished, and *Dbh* expression was now reduced. In conclusion, we found a much more global phenotype in the *Gata3*<sup>-/-</sup> mouse embryos than previously reported (Lim et al., 2000), which is also more in agreement with the effect of dn*Gata2* in chick sympathetic ganglia.

### ***Gata2* overexpression reveals a preferential action on generic neuron differentiation rather than on noradrenergic differentiation in peripheral nerve precursor cells**

The loss-of-function approaches revealed an essential role of *Gata2/3* in sympathetic neuron development but did not reveal the mechanism of action. The loss of sympathetic ganglion cells in *Gata2/3*-deficient ganglia may be explained by a selective control of cell survival by *Gata2/3* or by effects on differentiation that indirectly lead to apoptotic cell death. To begin to address these issues, the action of *Gata2* was studied in gain-of-function experiments. Using the chick embryo as a model to overexpress transcriptional control genes in neural crest precursor cells (Stanke et al., 1999; Howard et al., 2000; Stanke et al., 2004), we observed that *Gata2* induced the generation of ectopic neurons in peripheral nerve precursors (Fig. 9). These neurons expressed NF160 (*Nef3* – Mouse Genome Informatics) in addition to *SCG10* (not shown). Interestingly, the great majority of these ectopic neurons were devoid of the autonomic markers *Phox2a* and *Phox2b*. Only a small subpopulation of the *Scg10*-positive cells displayed properties of noradrenergic neurons, *Phox2b*, *Th*, *Dbh* (not shown) and *Cash1* (Fig. 9). *Cash1* expression was very low, which is expected from its transient expression during normal development. Double in-situ hybridisation for *Scg10* and *Th* demonstrated the very small proportion of the *Scg10*-positive cells that coexpressed *Th*. In addition to *Th*<sup>+</sup> neurons, there was also a small number of *Th*<sup>+</sup> cells devoid of neuronal properties. Infection of peripheral nerves with control RCAS virus [engrailed-RCAS-(BP)B] was unable to induce ectopic neurons (not shown), as expected from previous control RCAS infections (Reissmann et al., 1996; Stanke et al., 1999).

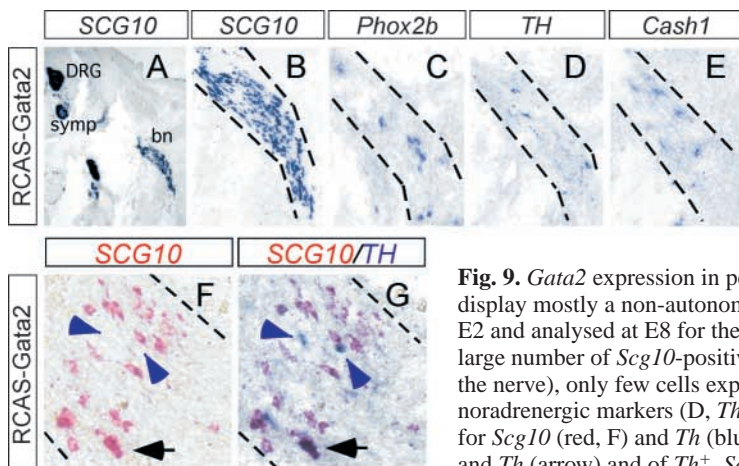
These data suggest a role for *Gata2* and *Gata3* in the neuronal differentiation of sympathetic precursor cells in the chick and mouse, respectively. The preferential generation of

non-autonomic neurons in response to *Gata2* in the peripheral nerve implies that the action of *Gata2* depends on the cellular context, i.e. that the effect on *Th* expression, revealed by the loss-of-function approaches, seems to be dependent on co-regulators present in the sympathetic neuron lineage but not in peripheral nerve precursor cells.

### ***Gata2* expression in parasympathetic ganglia and the locus coeruleus**

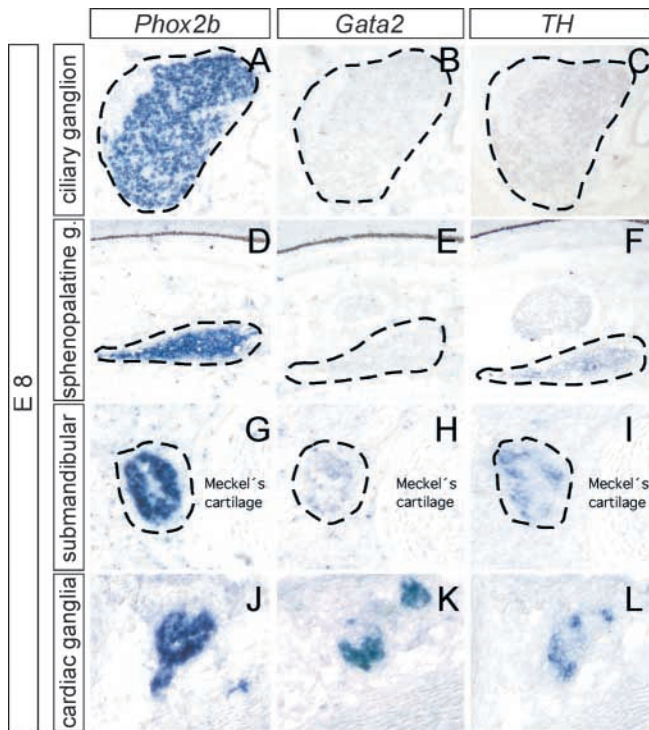
The importance of *Gata2/3* in sympathetic neuron development raised the issue whether *Gata2/3* would play a similar role in other autonomic ganglia and/or in central noradrenergic neurons. The parasympathetic chick ciliary ganglion was found to completely lack *Gata2* and *Gata3* expression at all stages analysed by in-situ hybridisation between stage 20 (E3) and stage 35 (E8) (Fig. 10). The result from the in-situ hybridisation was confirmed by semiquantitative RT-PCR, which revealed in E5 ciliary ganglia 1000-fold and 200-fold lower levels of *Gata2* and *Gata3* mRNA, respectively, compared with *Gata2* mRNA in E7 sympathetic ganglia. Low-level signals for *Gata2* and *Gata3* by RT-PCR can be explained by *Gata* expression in cells of the retro-orbital mesenchyme, contaminating to some extent the ganglion preparation. Also the chick parasympathetic sphenopalatine ganglion was devoid of *Gata2* expression (Fig. 10). However, *Gata2* was detectable in the submandibular ganglion (Fig. 10), and strong *Gata2* expression was present in trunk parasympathetic ganglia, i.e. cardiac ganglia (Fig. 10) and the Remak ganglion (Groves et al., 1995). From all chick ganglia investigated, *Gata3* was detected only in the cardiac ganglia (not shown). In the mouse, the sphenopalatine, otic and submandibular ganglia were devoid of *Gata3* expression at E13.5, with low, but detectable expression in cardiac ganglia (not shown). Interestingly, low-level *Th* expression was present in the chick parasympathetic sphenopalatine, submandibular, cardiac (Fig. 10) and the Remak ganglion (Cantino et al., 1982; Suzuki et al., 1994), whereas in the ciliary ganglion only very few *Th*-positive cells remained (Müller and Rohrer, 2002) (Fig. 10). *Dbh* expression paralleled *Th* expression, both with respect to ganglion type and expression levels (not shown). The expression of a variable subset of noradrenergic traits in cholinergic parasympathetic neurons has been described previously in several species (Grzanna and Coyle, 1978; Landis et al., 1987; Leblanc and Landis, 1989; Baluk and Gabella, 1990; Hardebo et al., 1992).

As *Gata2/3* are essential for the initiation and/or maintenance of *Th* and *Dbh* in noradrenergic sympathetic neurons it was of interest whether central noradrenergic neurons also depend on *Gata2/3* for their differentiation. The major noradrenergic centre of the central nervous system, the locus coeruleus, lacked *Gata2* and *Gata3* expression in mouse (Fig.



**Fig. 9.** *Gata2* expression in peripheral nerve precursors results in the generation of neurons that display mostly a non-autonomic phenotype. Chick embryos were infected by RCAS-*Gata2* at E2 and analysed at E8 for the expression of ectopic neurons in the brachial nerve. Whereas a large number of *Scg10*-positive cells was detected (A, overview; B, enlargement of the region of the nerve), only few cells expressing autonomic marker genes (C, *Phox2b*; E, *Cash1*) or noradrenergic markers (D, *Th*) were present. This is confirmed by double in-situ hybridisations for *Scg10* (red, F) and *Th* (blue, G). Please note the low number of cells that coexpress *Scg10* and *Th* (arrow) and of *Th*<sup>+</sup>, *Scg10*<sup>-</sup> cells (arrowheads).





**Fig. 10.** Expression of *Gata2* and *Th* in chick parasympathetic ganglia. Ciliary (A-C), sphenopalatine (D-F), submandibular (G-I) and cardiac (J-L) ganglia were analysed for the expression of *Phox2b*, *Gata2* and *Th*. Whereas *Gata2* is not detectable in the cranial ciliary and sphenopalatine ganglion, *Gata2* is expressed at very low levels in submandibular ganglia, and strong expression is evident in cardiac ganglia. Low-level *Th* expression is detectable in the sphenopalatine ganglion, submandibular and cardiac ganglia and in a small number of cells in the ciliary ganglion (Müller and Rohrer, 2002), but not in the section shown in C. Expression was analysed on parallel sections.

11) and chick embryos (not shown) and *Gata3* knockout mice had an intact locus coeruleus (Fig. 11). These results, together with the findings in parasympathetic ganglia, demonstrate that there is no strict correlation between *Gata2/3* expression and noradrenergic differentiation.

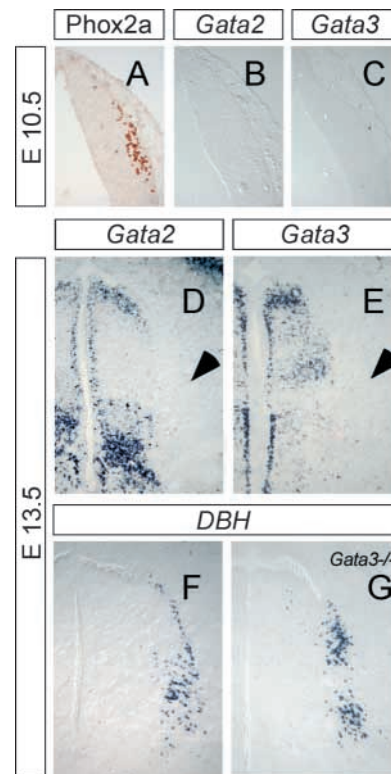
## Discussion

Here, we demonstrate that Gata transcription factors, *Gata2* in chick and *Gata3* in mouse, are members of the network of transcription factors controlling the development of noradrenergic sympathetic neurons from neural crest precursors. *Gata2* is expressed in a Bmp-dependent manner, immediately before the onset of noradrenergic differentiation, but after *Mash1*, *Phox2b*, *Hand2* and *Phox2a*. The *Phox2b*-dependent expression of *Gata2* and *Gata3*, evident in the *Phox2b* knockout, suggests a similar timing of expression in the mouse. Loss-of-function experiments demonstrate the importance of *Gata3* in the mouse and *Gata2* in the chick for sympathetic neuron development and for the onset of *Th* expression. *Gata2* overexpression in the chick embryo elicits the generation of ectopic neurons from neural crest precursor cells, which implicates a function of *Gata2/Gata3* in the

expression of generic and subtype-specific neuronal traits. The action of *Gata2/Gata3* depends, however, on the cellular context, as the vast majority of *Gata2*-induced ectopic neurons in the peripheral nerve display a non-autonomic, non-noradrenergic phenotype, whereas in sympathetic neurons *Th* expression and the maintenance (but not the onset) of *Dbh* expression are dependent on *Gata2/Gata3*. Thus, the effects on *Th* and *Dbh* may depend on additional co-regulators present in the sympathetic lineage.

### Gata factors in relation to other Bmp-induced transcriptional determinants of the sympathetic lineage

The members of the Gata family of zinc finger transcription factors have selective effects on various aspects of tissue and organ development. Of the six family members, only *Gata2* and *Gata3* are expressed in the vertebrate nervous system and were shown to control the development and differentiation of specific neuronal subpopulations (Yamamoto et al., 1990; Ko and Engel, 1993; Whyatt et al., 1993; Zhou et al., 2000; Karunaratne et al., 2002; Dasen et al., 1999; van Doorninck et al., 1999; Craven et al., 2004; Lim et al., 2000; Karis et al., 2001). In general, *Gata2* and *Gata3* are coexpressed in the central nervous system and *Gata3* is downstream of *Gata2* (Nardelli et al., 1999; Pata et al., 1999). The expression of these factors in the peripheral nervous system is much less clear, as



**Fig. 11.** *Gata2* and *Gata3* do not control the central noradrenergic phenotype. (A-C) The anlage of locus coeruleus (LC) visible in the dorsal metencephalon by anti-*Phox2a* immunohistochemistry (A) expresses neither *Gata2* (B) nor *Gata3* (C) at E10.5. (D-E) At E13.5 the anlage of the LC, visible by in-situ hybridisation for *Dbh* (F) expresses neither *Gata2* nor *Gata3* (arrowhead in D and E, respectively) and is intact in *Gata3* mutants (G).

data are often available for only one factor in a single species and tissue (Groves et al., 1995; Nardelli et al., 1999; George et al., 1994). We demonstrate here that *Gata3* is not expressed in chick sympathetic ganglia, whereas *Gata2* is detectable throughout development. The onset of *Gata2* expression in the chick was found to occur after the sequential expression of *Mash1*, *Phox2b*, *Hand2* and *Phox2a*.

The timing of *Gata2* expression and its proposed role in noradrenergic differentiation raised the question whether *Gata2* expression is dependent on Bmps, which have been shown to control the expression of *Mash1*, *Phox2a/b* and *Hand2* in sympathetic neuron precursors at the dorsal aorta (Schneider et al., 1999; Howard et al., 2000) and which control Gata factor expression in other developmental contexts (Maeno et al., 1996; Schultheiss et al., 1997; Rossi et al., 2001; Patient and McGhee, 2002). Alternatively, *Gata2* expression might be induced by additional, independent signals. The present data firmly establish that *Gata2* expression is prevented by the Bmp inhibitor noggin and is expressed in Bmp-induced ectopic neurons. Thus, *Gata2* represents an additional member of the group of transcription factors induced by Bmps. The epistatic relationship between *Gata2* and the other factors has been addressed by overexpression of *Phox2b*, *Hand2* and *Mash1*. The induction of *Gata2* expression by each of these factors is in agreement with the timing of expression and suggests that *Gata2* may be directly or indirectly controlled by these transcription factors. The notion that *Gata2* is expressed downstream of *Phox2b* is confirmed by the lack of *Gata2/3* expression in the *Phox2b* knockout mice and, conversely, the initial presence of cells expressing *Phox2b*, *Phox2a* and *Hand2* in the absence of *Gata3*.

### Generic and subtype-specific role of Gata factors in the sympathetic lineage

A major problem in the control of neurogenesis is how and when the expression of neuron subtype-specific properties and generic neuronal characteristics are coordinated. For sympathetic neuron development this issue is still unclear. The loss of *Mash1* and *Phox2b* does affect both noradrenergic and pan-neuronal gene expression (Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 1999) and in gain-of-function experiments no selective effects were observed for *Phox2a*, *Phox2b* and *Hand2* (Stanke et al., 1999; Howard et al., 2000). Under certain in-vitro conditions, *Mash1* was able to induce properties of autonomic neurons, but not noradrenergic differentiation (Lo et al., 1998). Also in vivo, *Mash1* overexpression in peripheral nerve precursors results in the preferential generation of non-adrenergic neurons, suggesting a major role of *Mash1* in the control of generic neuronal traits (Stanke et al., 2004). The previous analysis of *Gata3*-deficient mice suggested that *Gata3* may selectively control the noradrenergic phenotype in this lineage (Lim et al., 2000). To define the role of *Gata2* in chick sympathetic neurons, a dominant-negative variant of *Gata2* was expressed in developing sympathetic ganglia. The *Gata2* knockdown resulted in reduced Th expression and in a smaller size of sympathetic ganglia, identified by *Phox2b* and *Scg10*. In agreement with this action of dnGATA2 on ganglion size, we found a virtually complete loss of the superior cervical and stellate ganglia and the strong atrophy of thoracic sympathetic chain ganglia in *Gata3*<sup>-/-</sup> mice, not reported in the first

description of these mutants (Lim et al., 2000). In the cells of the rudimentary sympathetic ganglia the expression of Th is almost abolished and that of *Dbh* diminished.

In *Gata3*<sup>-/-</sup> embryos, expression of *Phox2b*, *Mash1* and *Dbh* were initially normal, while *Th* expression was already substantially reduced and that of *Gata2* virtually absent. The lack of *Gata2* expression is surprising, as *Gata2* has been shown to be upstream of *Gata3* in several systems. However, reciprocal crossregulations between *Gata2* and *Gata3* have also been observed in the spinal cord (Karunaratne et al., 2002) and hindbrain (Craven et al., 2004). The strong reduction in *Th* expression at E10.5 suggests a role for *Gata3* in the establishment of high-level *Th* expression. Already at E11.5 the sympathetic ganglion size is reduced and, in parallel, the number of apoptotic cells is strongly increased. The continued cell death is thought to result in the rudimentary sympathetic ganglia observed at E13.5.

These findings raise the question of how *Gata2/3* functions in sympathetic precursor cells and why the cells die in the absence of *Gata2/3*. *Gata2/3* factors could specifically control sympathetic neuron survival (but not differentiation) or, alternatively, control sympathetic neuron differentiation in a more general way. In the latter case, immature cells would be generated in *Gata3*<sup>-/-</sup> ganglia that subsequently die since they are deficient in many properties, including survival signalling. We favour the latter possibility, since in gain-of-function experiments *Gata2* acts as a differentiation factor rather than as a survival factor inducing the production of neurons in peripheral nerves, devoid of neurons during normal development.

The ectopic expression of *Phox2a*, *Phox2b* and *Hand2* in neural crest precursor cells elicits the generation of noradrenergic neurons (Stanke et al., 1999; Howard et al., 2000). This is explained by the strong crossregulations among these factors, resulting in the induction of the complete network by each individual factor. Thus, it was expected that *Gata2* may be able to induce the corresponding set of co-regulators required for noradrenergic and generic neuronal differentiation. The present results do not support this possibility and reveal for *Gata2* a potential to control generic neuronal differentiation. This indicates that the function of *Gata2* is dependent on the interaction with co-regulators, resulting in the induction of noradrenergic genes in the context of *Mash1*, *Phox2a/b* and *Hand2* in sympathetic precursors, while non-autonomic neurons are generated in peripheral nerve precursors. Whereas overexpression of *Phox2a* and *Hand2* induce upstream members of the transcriptional network involved in sympathetic neuron differentiation, *Gata2*, perhaps as the most downstream factor, has only a very weak crossregulating activity with respect to *Phox2a*, *Hand2*, *Phox2b* and *Cash1*.

What is the reason for the generation of a small population of noradrenergic, autonomic neurons and of some *Th*-positive cells devoid of *Scg10* in *Gata2*-infected nerves? The most likely explanation is that peripheral nerve precursors represent a mixture of cells at different stages of commitment and differentiation. Only in a minor fraction of the cells *Gata2* may be able to induce *Phox2a* and additional upstream transcription factors that would elicit, together with *Gata2*, noradrenergic neuron development. It should be noted that peripheral nerve precursors are biased towards autonomic neuron differentiation

(White et al., 2001). *Th*-positive, *Scg10*-negative cells might be explained by the very low *Cash1* expression in *Gata2* overexpression experiments.

### Gata function and the noradrenergic phenotype

In the autonomic nervous system, *Mash1* and *Phox2* transcription factors are essential for the generation of both sympathetic and parasympathetic ganglion neurons, i.e. functionally noradrenergic and cholinergic phenotypes (for the most part). Therefore, additional regulators have to be hypothesized which modify *Phox2* and *Mash1* action and are selectively expressed in noradrenergic or cholinergic neurons. There is evidence that the bHLH transcription factor *Hand2* is such a factor: it is expressed selectively in sympathetic neurons and capable, upon ectopic expression, of inducing adrenergic differentiation in neural crest precursors and to maintain the normally transient *Th* expression of parasympathetic ciliary neurons (Müller and Rohrer, 2002). The present observations identify another such factor in the form of *Gata2/3*, also absent from ciliary and sphenopalatine parasympathetic ganglia, and which, in combination with *Hand2* (and possibly in direct interaction with it (Dai et al., 2002)) may contribute to the continued expression of *Th* and *Dbh* in sympathetic neurons. However, investigation on a larger scale shows that *Gata2/3* function is not associated with noradrenergic properties per se. Although *Gata2* expression in the parasympathetic cardiac, submandibular and Remak's ganglion (Groves et al., 1995) correlates with the presence of noradrenergic gene expression, *Gata2* and *Gata3* are not expressed in the chick and mouse sphenopalatine ganglion, also containing considerable numbers of neurons expressing *Th* and/or *Dbh* (Fig. 10). It should be mentioned in this context that variable aspects of noradrenergic traits are expressed in cholinergic parasympathetic neurons, often transiently, and never resulting in a functionally noradrenergic phenotype (Grzanna and Coyle, 1978; Landis et al., 1987; Leblanc and Landis, 1989; Baluk and Gabella, 1990; Hardebo et al., 1992). Finally, *Gata2/3* are absent in both chick and mouse from the major noradrenergic centre of the brain, the locus coeruleus, and the development of the locus coeruleus does not depend on *Gata3*. The selective function of *Gata2/3* in the development of noradrenergic sympathetic but not LC neurons illustrates differences in the molecular control of the noradrenergic phenotype in different lineages, after the initial, common dependence on *Mash1* and *Phox2a/b*.

In conclusion, *Gata2/3* have been identified as members of the group of *Hand2*-induced transcription factors that are essential for the generation and differentiation of sympathetic neurons. Among the sympathetic phenotypic traits that were tested to date, *Gata2/Gata3* displays a preferential role in the expression of *Th*, a function that depends, however, on the presence of additional co-regulators present in the sympathetic neuronal lineage. It will be interesting to investigate whether *Phox2a/b* and *Hand2* and/or unknown co-regulators are physically interacting with *Gata2/3* and to identify the target genes controlled by *Gata2/3* in the sympathetic lineage.

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